IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Filed: September 15, 2003 Examiner: Raymond J. Henley, III

Application No.: 10/662,975 Group: 1614

Confirmation No.: 2253

For: METHOD OF USING PYRUVATE AND/OR ITS DERIVATIVES FOR THE TREATMENT OF CYTOKINE-MEDIATED INFLAMMATORY CONDITIONS

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as First Class Mail in an envelope addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450

on 11-1-04 Donna Bartolone
Date Signature

Donna Bartolone
Typed or printed name of person signing certificate

DECLARATION OF MITCHELL P. FINK UNDER 37 C.F.R. 1.132

Commissioner of Patents and Trademarks
P.O. Box 2327
Alexandria, VA 22202

Sir:

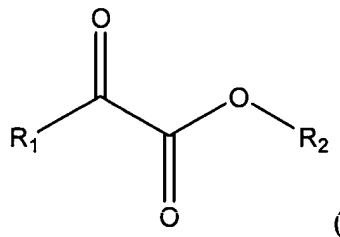
I, Mitchell P. Fink, Ph.D., of 109 Rockwood Drive, Pittsburgh, PA, 15238, declare and state that:

1. I am an inventor of the above subject matter described in U.S. Serial No. 10/662,975, claiming a method for treating a cytokine-mediated inflammatory condition in a patient suffering therefrom. In a first embodiment, the method

comprises the step of administering to the patient an effective amount of a composition comprising an ester of an alpha-ketoalkanoic acid in a pharmaceutically acceptable inert carrier substance.

In this first embodiment, the condition can be selected from the group consisting of rheumatoid spondylitis, osteoarthritis, gouty arthritis, endotoxic shock, cerebral malaria, silicosis, pulmonary sarcoidosis, bone resorption disease, graft versus host disease, allograft rejections, fever and myalgia due to infection, AIDS related complex (ARC), Crohn's disease, rheumatoid arthritis, cachexia and septic shock.

In a second embodiment, a subject with an inflammatory condition is administered a compound of Formula (I) in a pharmaceutically acceptable inert carrier:



(I),

wherein R_1 is methyl, ethyl, propyl, isopropyl, n-butyl, sec-butyl, isobutyl, tert-butyl, pentyl, 4-methylpentyl, 3-methylpentyl, hexyl, heptyl, octyl, 1-phenylmethyl or 2-phenyl-ethyl; R_2 is ethyl, propyl, isopropyl, n-butyl, sec-butyl, isobutyl, tert-butyl, pentyl, 4-methylpentyl, ethoxymethyl, 2-ethoxyethyl, carboxymethyl or ethoxycarbonylmethyl.

2. I graduated with a Doctor of Medicine degree from the Washington University in St. Louis, Missouri (1976), and completed a Medicine Residency in Surgery at the National Naval Center in Bethesda, Maryland (1981). Currently, I am a Watson Professor of Surgery and the Chairman of the Department of Critical Care Medicine at the University of Pittsburgh. I have been working in the field of cytokine-mediated diseases since about 1982.

3. I have thoroughly studied the above-identified application and the Office Action mailed from the Patent Office on July 1, 2004. In the Office Action, the Examiner rejects Claims 1, 2, 4-13, 15-17 and 19-31 as allegedly being obvious over U.S. Patent No. 5,798,388 by Katz in view of U.S. Patent Application Publication No. 2003/0073,743 by Ajami *et al.* Katz teaches the use of pyruvic acid and pyruvic acid precursors for treating inflammatory disorders. Ajami teaches the use of esters of pyruvate for treating ischemic conditions. The Examiner further states that the use of simple alkyl esters of pyruvic acid would have been immediately appreciated by the skilled artisan for inflammation in view of the combined teachings of these two references.

4. Experiments carried out under my supervision have demonstrated that the ethyl ester of pyruvic acid is significantly more effective than pyruvic acid salts in providing protection against LPS-induced ileal mucosal hyperpermeability, bacterial translocation, and hepatocellular injury in mice. These experiments are described in the Sections below.

5. ETHYL PYRUVATE PROVIDES BETTER PROTECTION THAN PYRUVATE AGAINST LPS-INDUCED GUT BARRIER DYSFUNCTION AS MEASURED BY ILEAL MUCOSAL HYPERPERMEABILITY, INDUCTION OF *iNOS* EXPRESSION AND INCREASED PRODUCTION OF *NO·*.

Intestinal hyperpermeability refers to impairment of the intestinal mucosal barrier, which is central to healthy absorption of nutrients and protection against bacterial and toxin translocation from the gastrointestinal (GI) tract to the blood stream or other organs. Disturbances in mucosal barrier function contribute to inflammatory conditions or systemic infection. Increased mucosal permeability occurs as an early response to a developing inflammation. Increased permeability of ileal tissues allows an influx of leukocytes into the intestine in response to inflammation, leading to submucosal tissue damage. Thus, intestinal permeability is used as a measure of the ability of a compound to block the inflammatory response of a subject after exposure to an inflammatory stimulus.

Nitric oxide (NO) is an inorganic, gaseous free radical that carries a variety of messages between cells. NO production is mediated by members of the nitric oxide synthase (NOS) family. One form of NOS is an inducible isoform iNOS. The expression of iNOS is induced by the actions of cytokines (e.g., tumor necrosis factor, various interleukins) and bacterial endotoxins (e.g., lipopolysaccharide). Induction of iNOS results in NO production. This is an important mechanism in the pathogenesis of inflammation. See Unno N., *et al. Gastroenterology* 113:1246-1257, 1997 and Mishima S., *et al. Arch Surg* 132:1190-1195, 1997.

Induction of iNOS expression and increased production of NO, measurable by concomitant increases in NO_3^- and NO_2^- concentration, have been implicated as important factors contributing the development of gut barrier dysfunction following the injection of rodents with LPS. Thus, ability of a compound to inhibit cytokine-induced induction of iNOS expression and NO_3^- /NO_2^- concentration are used as a measure of the ability of this compound to block the inflammatory response of a subject after exposure to an inflammatory stimulus.

Materials and methods

Animals.

The research protocol complied with the regulations regarding animal care as published by the National Institutes of Health and was approved by the Institutional Animal Use and Care Committee of the University of Pittsburgh Medical School. Male C57Bl/6J mice weighing 20-25 g (Jackson Laboratories, Bar Harbor, ME) were maintained at the University of Pittsburgh Animal Research Center with a 12-hour light-dark cycle and free access to standard laboratory fed and water. Animals were not fasted prior to the experiments.

Materials.

All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless otherwise noted. Dulbecco's modified Eagle medium (DMEM) and PBS were from BioWhittaker (Walkersville, MD). Fetal bovine serum (FBS; < 0.05 endotoxin units per ml) was obtained from Hyclone (Logan, UT). Recombinant IFN-γ, TNF-α, and IL-1β were obtained from Pierce-Endogen (Rockford, IN). Caco-2 human intestinal epithelial cells were obtained from the American Type Culture Collection (Manassas, VA).

Experimental designs for in vivo experiments.

In the *in vivo* experiments, five groups of mice (N=5 each) were studied. All agents were injected intraperitoneally.

Mice in the "No Rx" group (i.e., the positive control group), were injected with four 0.3 ml doses of Ringer's Lactate Solution (RLS) during the interval from T= -24 to -6 h. At T=0 h, these mice were challenged with 1.0 ml of a well-sonicated suspension of *Escherichia coli* serotype 0111:B4 LPS (0.1 mg/ml; 4 mg/kg) in PBS. Six and twelve hours later, these mice were injected with 0.31 ml of Ringer's Lactate Solution (RLS).

Mice in the "EP pre/pst Rx" group (i.e., ethyl pyruvate (EP) pre- and post-treatment group) were injected with LPS at T=0 h and with six doses of EP solution (T=-24, -18, -12, -6, +6, and +12 h).

Mice in the "Pyr pre/pst" group (i.e., the sodium pyruvate group) were injected at T=-24, -18, -12, -6, +6, and +12 h with 0.3 ml of a freshly-prepared solution containing 109 mM NaCl, 4.0 mM KCl, 2.7 mM $CaCl_2$, and 28 mM sodium pyruvate. This composition provided the amount of pyruvate equimolar to the amount of ethyl pyruvate received by the "EP pre/pst" group. At T=0 h, these mice were challenged with LPS.

Eighteen hours after the injection of lipopolysaccharide (LPS) (or the PBS vehicle in the No Rx groups), the mice were anesthetized with an intramuscular injection of sodium pentobarbital (90 mg/kg), and segments of ileum were excised for determination of mucosal permeability (see FIG. 1). Liver was harvested for

mRNA expression of iNOS and blood was aspirated from the heart to measure the plasma concentrations of NO_2^-/NO_3^- (see FIG. 2B).

Measurement of intestinal barrier function.

Intestinal mucosal permeability to the fluorescent tracer, FD4, was determined using an everted gut sac method, exactly as described previously by Yang et al., in "Ethyl pyruvate modulates inflammatory gene expression in mice subjected to hemorrhagic shock." *Am J Physiol Gastrointest Liver Physiol* 283:G212-G22 (2002). Quantitation of translocation of viable bacteria to MLN was also determined as described previously by Yang et al. in "Ethyl pyruvate modulates inflammatory gene expression in mice subjected to hemorrhagic shock." *Am J Physiol Gastrointest Liver Physiol* 283:G212-G22 (2002).

Measurement of iNOS expression levels using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR).

To estimate iNOS mRNA levels, total RNA was extracted from Caco-2 cells or mouse hepatic tissue samples with chloroform and TRI Reagent (Molecular Research Center, Cincinnati, OH) as directed by the manufacturer. The total RNA was treated with DNAFree (Ambion, Houston, TX) as instructed by the manufacturer using 10 units of DNase I per 10 μg RNA. Two μg of total RNA was reverse transcribed in a 40 μl reaction volume containing 0.5 μg of oligo$(dT)_{15}$ (Promega), 1 mM of each dNTP, 15 U AMV reverse transcriptase (Promega), and 1 U/μL of RNasin ribonuclease inhibitor (Promega) in 5 mM $MgCl_2$, 50 mM KCL, 0.1% Triton X-100, 10 mM Tris-HCl (pH 8.0). The reaction mixture was preincubated at 65 °C for 10 min prior to DNA synthesis. The RT reaction was carried out for 50 min at 42 °C and was heated to 95 °C for 5 min to terminate the reaction. Reaction mixtures (50 μL) for PCR were assembled using 5 μL of cDNA template, 10 units AdvanTaq Plus DNA Polymerase (Invitrogen, Carlsbad, CA), 200 μM of each dNTP, 1.5 mM $MgCl_2$ and 1.0 μM of each primer in 1× AdvanTaq Plus PCR buffer. The PCR reactions were performed using a Perkin Elmer Model 480 thermocycler (Norwalk, CT).

Amplification of iNOS cDNA was carried out as previously described. See Yang R. *et al. Am J Physiol Gastrointest Liver Physiol* 283:G212-G22 (2002) and Sappington P.L. *et al. J Pharmacol Exp Ther* 304:464-476 (2003).

18S ribosomal RNA was amplified to verify equal loading as described above. Ten μl of each PCR reaction was electrophoresed on a 2 % agarose gel in 1× Tris-acetate-EDTA (TAE) buffer, scanned in NucleoVision imaging workstation (NucleoTech, San Mateo, CA), and quantified using GelExpert™ release 3.5. Results are expressed as relative band densities, calculated by dividing the density of iNOS band by the density of the corresponding 18S band.

Measurement of NO· production.

To determine the concentration of NO_3^- plus NO_2^-, the end-products of NO· metabolism, aliquots of serum were cleared by centrifugation. NO_2^-/NO_3^- concentrations in serum or culture supernatants were quantitated using the Bioxytech Nitric Oxide Assay Kit exactly as directed by the manufacturer (OXIS International, Inc. Portland, OR).

Results

As shown in FIG. 1, pre- and post-treatment with either EP or sodium pyruvate significantly ameliorated LPS-induced ileal mucosal hyperpermeability to FD4. However, EP was significantly more effective in this regard than was sodium pyruvate. In fact, administration of ethyl pyruvate reduced ileal mucosal hyperpermeability by almost a factor of 10 compared to administration of sodium pyruvate.

FIGs. 2A and 2B show comparison of the effects of ethyl pyruvate versus sodium pyruvate on hepatic iNOS mRNA expression (FIG. 2A; n=3 per condition) and plasma NO_2^- plus NO_3^- concentrations (FIG. 2B) in endotoxemic mice. A representative gel is depicted in the inset. Each lane corresponds to one of the groups depicted on the accompanying bar graphs; from left to right, the order of the groups is same for the bar graphs and the gel. Results are means ± SE. * indicates $p<0.05$ versus RLS and † indicates $p<0.05$ versus No Rx.

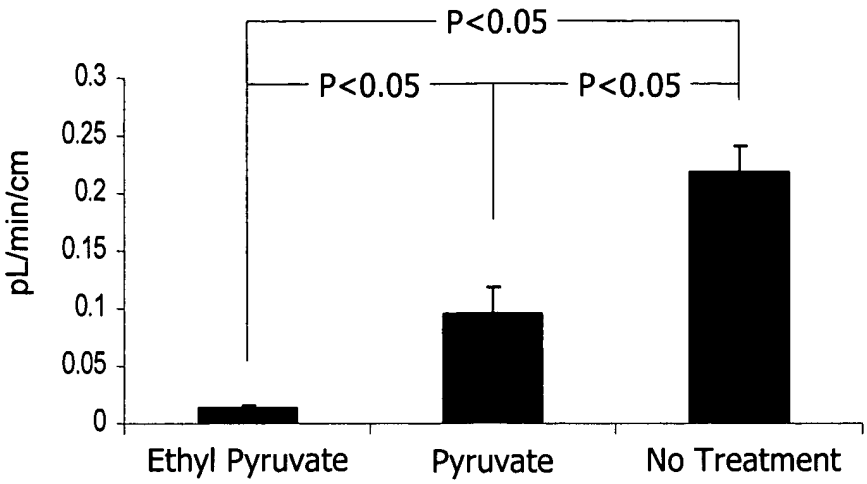


FIG 1. Ileal Mucosal Permeability in Mice Challenged with LPS

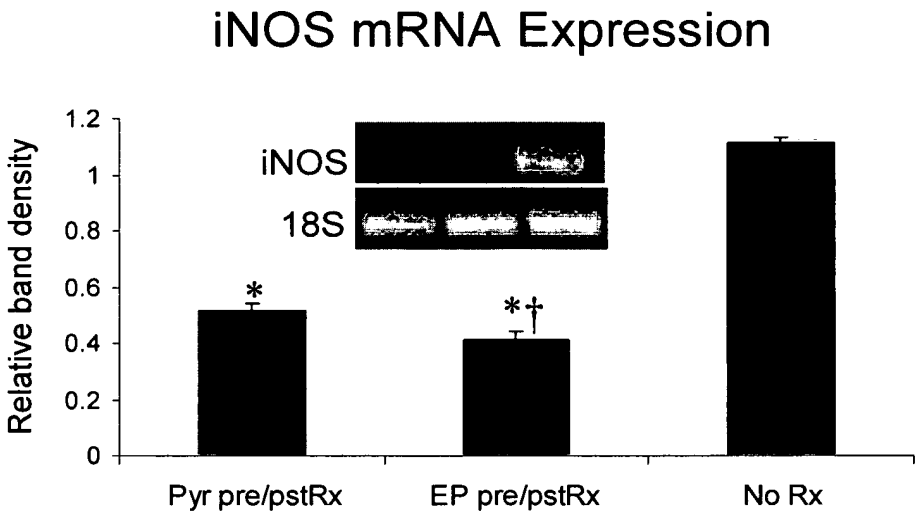


FIG. 2A

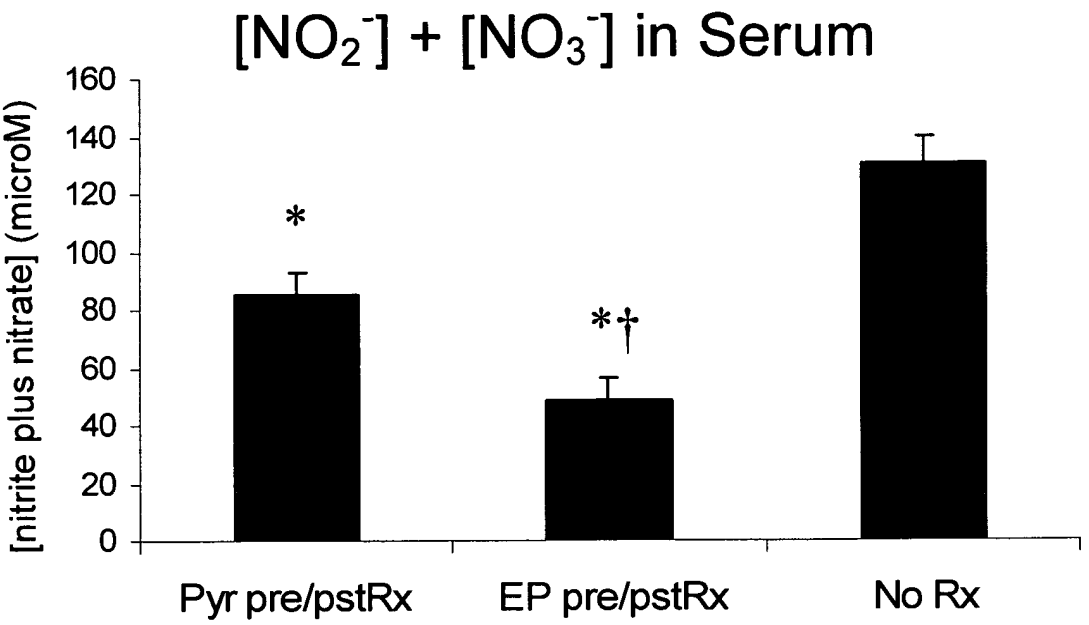


FIG. 2B

Ethyl pyruvate is more effective then sodium pyruvate in reducing *iNOS* mRNA level of expression and *NO* concentration.The difference between levels of inhibition of iNOS expression by ethyl pyruvate and sodium pyruvate was statistically significant, $p < 0.05$.

6. ETHYL PYRUVATE IS SIGNIFICANTLY MORE EFFECTIVE IN INHIBITING CYTOKINEINDUCED ICAM-1 (CD 54) EXPRESSION THAN SODIUM PYRUVATE

Cytokine-induced expression of cellular adhesion molecules is an early indicator of inflammation. For example, InterCellular Adhesion Molecules (ICAMs) and vascular cell adhesion molecule-1 (VCAM-1) are part of the immunoglobulin superfamily. They are important in inflammation, immune responses and in intracellular signalling events. The ICAM family, which consists of five members, designated ICAM-1 to ICAM-5, is known to bind to leukocyte integrins CD11/CD18 during inflammation and in immune responses. ICAM-1 (CD54) is expressed on leukocytes, endothelial and epithelial cells, and is upregulated in response to challenges to immune system such as bacterial invasion. It is believed to be involved in recruitment of leukocytes to the

inflamed tissues. Thus, ability to inhibit cytokine-induced induction of ICAM-1 expression is used as a measure of the ability of this compound to block the inflammatory response of a subject after exposure to an inflammatory stimulus.

Materials and Methods

Cell Culture

The U373 astrocytoma cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA), and it was maintained at 37 °C in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% pyruvate, and non-essential amino acid supplement (1% v/v). Cells were passed once a week and used for experiments at 90% confluence.

Fluorescence Activated Cell Sorting (FACS) enrichment protocol

The parental U373 cell line was incubated in the presence of IL-1β (10 ng/ml) for 18 hours (h). The cells were washed with DMEM and then incubated with anti-human CD54-FITC (CALTAG, Burlingame, CA) to dectect expression of CD54 (ICAM-1). Cells were fixed with 2% paraformaldehyde in PBS and analyzed by flow cytometry on a FACSVantage Cell Sorter.

Cells with greater than basal fluorescence after exposure to IL-1β (10 ng/ml) were collected using a BD FacsVantage Cell Sorter. The sorted cells were plated into T25 flasks and individual colonies cloned. When the bulk population of parental strain U373 cells was exposed to IL-1β only 50-60% of the cells expressed CD54. However, we were able to select subclones that were more responsive to stimulation with IL-1β. Thus, in various subclones, 70-98% of the cells expressed CD54 after incubation with IL-1β. The most responsive of these subclones was used for all further studies.

Flow Cytometry Analysis

Cells were cultured as described above for 18 h in the presence or absence of IL-1β in the absence or presence of one of various concentrations of ethyl pyruvate or sodium pyruvate. Cells were harvested with 10 mM EDTA in PBS. Cells were washed with DMEM and then incubated with anti-human CD54-FITC.

Cells were fixed with 2% paraformaldehyde in PBS and analyzed by flow cytometry on a FACSVantage Cell Sorter.

Pharmacologic Analysis Using FACS

Percent inhibition for each compound was determined by comparing the mean channel fluorescence (MCF) of the cells stimulated with IL-1β with the MCF of the cells which were pretreated with the compound of interest prior to treatment with IL-1β according to the formula:

percent inhibition = 1 - (MCF of the sample - MCF of unstimulated cells)/(MCF of IL-1β treated cells –MCF of unstimulated cells).

Results

As shown in FIG. 3, ethyl pyruvate showed remarkable efficacy in inhibiting IL-1β-induced ICAM-1 expression. In contrast, sodium pyruvate exhibited no activity. These results indicate that ethyl pyruvate, but not pyruvic salts, are effective in suppression of inflammatory responses mediated by ICAM-1.

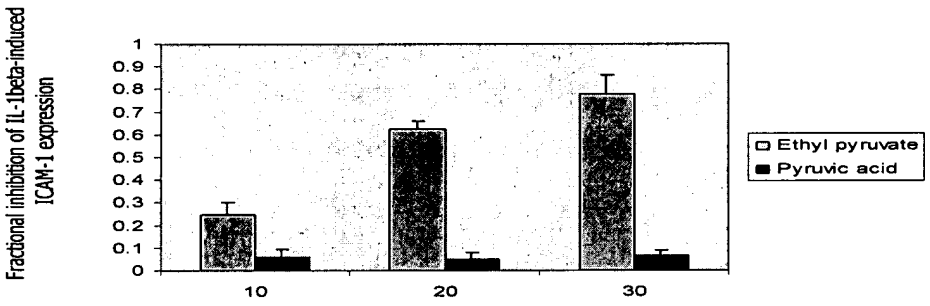


FIG. 3

7. Based on the data presented in Sections 5 and 6, it is evident that pyruvyl esters have surprisingly better activities that are superior to pyruvate in the treatment of LPS-induced ileal mucosal hyperpermeability, bacterial translocation, and hepatocellular injury *in vivo* and cytokine-induced ICAM-1 expression *in vitro*.

Specifically, as demonstrated in Section 5, ethyl pyruvate exhibited surprisingly better efficacy than sodium pyruvate in reducing ileal mucosal permeability in LPS-challenged mice (FIG. 1) and was also more effective than sodium pyruvate in decreasing LPS-induced bacterial translocation to the MLN complex, and reducing circulating levels of ALT after the administration of LPS. As demonstrated in Section 6, ethyl pyruvate was remarkably more effective than sodium pyruvate in reducing the expression levels of ICAM-1 in cells treated with IL-1β. In fact, as shown in FIG. 3, sodium pyruvate exhibited virtually no anti-inflammatory activity in this assay system.

8. I hereby acknowledge that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Mitchell Fink

Mitchell P. Fink

Nov 1, 2004

Date